CELLOBIOHYDROLASE I GENE AND IMPROVED VARIENTS

Technical Field.

This invention relates to 1,4- β-cellobiohydrolases or exoglucanases. More specifically, it relates to the *Trichoderma reesei* cellobiohydrolase I gene, the creation of reduced glycosylation variants of the expressed CBHI protein to enable the expression of active enzyme in heterologous hosts, and to the creation of new thermal stabile variants of the enzyme that instill higher thermal tolerance on the protein and improved performance.

Background Art.

The surface chemistry of acid pretreated-biomass, used in ethanol production, is different from that found in plant tissues, naturally digested by fungal cellulase enzymes, in two important ways: (1) pretreatment heats the substrate past the phase-transition temperature of lignin; and (2) pretreated biomass contains less acetylated hemicellulose. Thus, it is believed, that the cellulose fibers of pretreated-biomass are coated with displaced and modified lignin. This alteration results in a non-specific binding of the protein with the biomass, which impedes enzymatic activity. Moreover, where the pretreated biomass is a hardwood-pulp it contains a weak net-negatively charged surface, which is not observed in native wood. Therefore, for the efficient production of ethanol from a pretreated biomass such as corn stover, wood or other biomass it is desirable to enhance the catalytic activity of glycosyl hydrolases specifically the cellobiohydrolases.

Trichoderma reesei CBH I is a mesophilic cellulase which plays a major role in the hydrolysis of cellulose. An artificial ternary cellulase system consisting of a 90:10:2 mixture of *T. reesei* CBH I, *Acidothermus cellulolyticus* EI, and *Aspergillus niger* β-D-glucosidase is capable of releasing as much reducing sugar from pretreated yellow poplar as the native *T. reesei* system after 120 h. This result is encouraging for the ultimate success of engineered cellulase systems, because this artificial enzyme system was tested at 50°C, a temperature far below that considered optimal for EI, in order to spare the more heat labile enzymes CBH I and β-D-glucosidase. To increase the efficiency of such artificial enzyme systems it is desirable to engineer new *T. ressei* CBH I variant enzymes capable of active expression in heterologous hosts. The use of the heterologous host *Aspergillus awamori*, could provide an excellent capacity for synthesis and secretion of *T. reesei* CBH I because of its ability to correctly fold and post-

translationally modify proteins of eukaryotic origin. Moreover, *A. awamori* is believed to be an excellent test-bed for Trichoderma coding sequences and resolves some of the problems associated with site directed mutagenesis and genetic engineering in Trichoderma.

In consideration of the foregoing, it is therefore desirable to provide variant cellulase enzymes having enzymatic activity when expressed in a heterologous host, and to provide variant cellulase enzymes that have improve thermal tolerance over the native as produced by *Trichoderma reesei*.

Disclosure of Invention.

It is a general object of the present invention to provide variant cellulase enzymes having enzymatic activity when expressed in a heterologous host, such as a filamentous fungi or yeast.

Another object of the invention is to provide a variant exoglucanases characterized by a reduction in glycosylation when expressed in a heterologous host.

Another object of the invention is to provide an active cellobiohydrolase enzyme capable of expression in heterologous fungi or yeast.

Another object of the invention is to provide improved thermal tolerant variants of the cellobiohydrolase enzyme capable of functioning at increased process temperatures.

It is yet another object of the invention to provide a method for reducing the glycosylation of a cellobiohydrolase enzyme for expression in a heterologous host.

The foregoing specific objects and advantages of the invention are illustrative of those which can be achieved by the present invention and are not intended to be exhaustive or limiting of the possible advantages which can be realized. Thus, those and other objects and advantages of the invention will be apparent from the description herein or can be learned from practicing the invention, both as embodied herein or as modified in view of any variations which may be apparent to those skilled in the art.

Briefly, the invention provides a method for making an active cellobiohydrolase in a heterologous host, the method comprising reducing glycosylation of the cellobiohydrolase, reducing glycosylation further comprising reducing an N-glycosylation site amino acid residue with a non-glycosyl accepting amino acid residue. The invention further provides a cellobiohydrolase, comprising the reduced glycosylation variant cellobiose enzymes CBHI-N45A; CBHI-N270A; or CBHI-N384A, or any combination thereof,

Best Mode for Carrying out the Invention.

Unless specifically defined otherwise, all technical or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described.

A method for reducing the glycosylation of an expressed Trichoderma reesei CBHI protein by site-directed mutagenesis ("SDM") is disclosed. The method includes replacing an N-glycosylation site amino acid residue, such as asparagines 45, 270, and/or 384 (referenced herein as CBHI-N45A, CBHI-N20A and CBHI-N384A, respectively), with a non-glycosyl accepting amino acid residue, such as is alanine. Various mutagenesis kits for SDM are available to those skilled in the art and the methods for SDM are well known. The description below discloses a procedure for making and using CBHI variants: CBHI-N45A; CBHI-N270A; and CBHI-N384A. The examples below demonstrate the expression of active CBHI in the heterologous fungus *Aspergillus awamori*.

Variants of CBH I embodiments that include mutations that provide for improved end product inhibition and for thermal tolerance.

Brief Description of the Figures.

Figure 1. Coding sequence for the *cbh1* gene. Small case letters represent the signal sequence, large case letters the catalytic domain, bolded italics the linker region, and large case underlined the cellulose-binding domain.

Figure 2. SDS-PAGE Western blot with anti-CBH I antibody showing the reduction on molecular weight of rCBH I expression clones as a function of introduction of N to A modifications.

Figure 3. Plasmid map for the fungal expression vector pPFE2/CBH I.

Figure 4. Coding sequence for the linker region showing additional proline that effect conformation of linker region in the protein structure.

Example 1. Acquisition of the CBH I Encoding Sequence.

Acquisition of the gene by was done by either cDNA cloning or by PCR of the gene from genomic DNA. CBH I cDNA was isolated from a *T. reesei* strain RUT C-30 cDNA library constructed using a PCR-generated probe based on published CBH I gene sequences (Shoemaker, et al., 1983). The cDNA's were cloned (using the Zap Express cDNA kit from Stratagene; cat. #200403) into the XhoI and EcoRI site(s) of the supplied, pre-cut lambda arms. An XhoI site was added to the 3' end of the cDNA during cDNA synthesis, and sticky-ended RI linkers were added to both ends. After XhoI digestion, one end has an XhoI overhang, and the other (5' end) has an Eco RI overhang. The insert can be removed from this clone as an approximately 1.7 kb fragment using SalI or SpeI plus XhoI in a double digest. There are two Eco RI, 1 one Bam HI, 3 SacI and one HindIII sites in the coding sequence of the cDNA itself. The plasmid corresponding to this clone was excised *in vivo* from the original lambda clone, and corresponds to pB210-5A. Thus, the cDNA is inserted in parallel with a Lac promoter in the pBK-CMV parent vector. Strain pB210-5A grows on LB + kanamycin (50 ug/mL).

Acquisition of the *cbh1* gene was also achieved by PCR of genomic DNA. With this approach the fungal chromosomal DNA from *T. reesei* strain Rut C-30 was prepared by grinding the fungal hypae in liquid nitrogen using a mortar and pestle to a fine powder. The genomic DNA was then extracted from the cell debris using a Qiagen DNAeasy Plant Mini kit. Amplification of the DNA fragment that encodes for the *cbh1* gene, including introns, was performed using polymerase chain reaction (PCR) with specific primers for the *T. reesei cbh1* gene. The primers 5'-AGAGAGTCTAGACACGGAGCTTACAGGC-3' that introduces a Xba I site and the primer 5'-AAAGAAGCGCGGCCGCGCCTGCACTCTCCAATCGG-3' that introduces a unique Not I site were used to allowing cloning into the pPFE *Aspergillus/E. coli*

shuttle vectors that are described below. The amplified PCR product was then gel purified and cloned directly into the vectors.

Example 2. Production of Active Recombinant CBHI (rCBH I) in *Aspergillus awamori*. Construction of the Fungal Expression Vectors pPFE-1/CBH1 and pPFE-2/CBH1.

The coding sequence for *T. reesei* CBH I was successfully inserted and expressed in *Aspergillus awamori* using the fungal expression vector pPFE2 (and pPFE1). Vectors pPFE1 and pPFE2 are *E. coli - Aspergillus* shuttle vectors, and contain elements required for maintenance in both hosts. Both pPFE-1 and pPFE-2 vectors direct the expression of a fusion protein with a portion of the glucoamylase gene fused to the gene of interest. The pPFE1 vector contains a region of the glucoamylase gene, with expression under the control of the *A. awamori* glucoamylase promoter. The protein of interest is expressed as a fusion protein with the secretion signal peptide and 498 amino acids of the catalytic domain of the glucoamylase protein. The majority of the work presented here was done using the pPFE2 expression vector, which was chosen because of its smaller size, simplifying the PCR mutation strategy by reducing extension time.

The major features of the pPFE2-CBH1 construct are shown in Figure 3. With both the pPFE1/CBH1 and the pPFE2/CBH1 vectors, the sequence immediately upstream of the Not I site encodes a LysArg dipeptide. A host KEX-2 like protease recognizes this dipeptide sequence during the secretion process, and the fusion peptide is cleaved, removing the glucoamylase secretion signal peptide or the longer catalytic domain of glucoamylase in the case of pPFE1. In this way, the recombinant CBH I protein experiences an "efficient ride" through the *A. awamori* secretion system and is expressed with the native N-terminal protein. The net result is that the recombinant CBH I is processed so that it can accumulate in the medium without its glucoamylase secretion signal fusion partner. The vector contains the *Streptoalloteichus hindustanus* phleomycin resistance gene, under the control of the *A. niger* β-tubulin promoter, for positive selection of *Aspergillus* transformants. The pPFE/CBH1 vector also contains a β-lactamase gene for positive selection using ampilcillin in *E. coli*, and also contains the *A. niger* trpC terminator. The insertion of the CBH I coding sequence into the pPFE vectors was accomplished using two methods. Vector DNA was first produced in 500 mL cultures of *E. coli* XL1 Blue and the plasmids purified using Promega maxi-preps DNA purification kits.

Approach 1: Blunt-Xba I fragment generation.

1. Oligonucleotides were designed to give a blunt end on the 5' end and an engineered Xba I site on the 3'end of the PCR fragment.
2. The full-length coding sequence for CBH I was obtained by PCR using Pfu DNA polymerase and using the cDNA construct pB510-2a as the template. Pfu DNA polymerase generates blunt-ended PCR products exclusively.
3. The pPFE vectors were digested using NotI and confirmed by agarose gel electrophoresis. The NotI overhang was then digested using Mung Bean nuclease. The DNA was purified and the vector and CBHI PCR fragment digested using XbaI.
4. The vector and PCR product were then ligated using T4 DNA ligase and the DNA used to transform *E. coli* XL-1 Blue and *E. coli* DH5α using electroporation.

Approach 2: NotI-XbaI fragment approach.

1. Oligonucleotides were designed to give a Not 1 site on the 5' end, and an engineered Xba I site on the 3'end of the PCR fragment.
2. The full-length coding sequence for CBH I was obtained by PCR using Pfu DNA polymerase and using the cDNA construct pB510-2a as the template.
3. The pPFE vectors and the PCR product were digested using Not 1 and Xba 1
4. The CBH I PCR product was directionally cloned into the pPFE2 vector using T4 DNA ligase and transformed into *E. coli* XL-1 Blue.
5. The insertion of the CBH I coding sequence into the pPFE2 vector was confirmed using PCR, restriction digest analysis, and DNA sequencing through the insertion sites. The entire coding sequence of the insert was also confirmed by DNA sequencing.

The constructs produced using these two methods was then used to transform *A. awamori* and to express rCBH I, as confirmed by western blot analysis of culture supernatant. The rCBH I expressed in *A. awamori* tends to be over glycosylated as evidenced by the higher molecular weight observed on western blot analysis. Over glycosylation of CBH I by *A. awamori* was confirmed by digestion of the recombinant protein with endoglycosidases. Following endoglycosidase H and F digestion, the higher molecular weight form of the protein collapses to a molecular weight similar to native CBH I.

Example 3. Method for producing PCR site directed mutations for glycosylation removal and improved thermalstability.

The QuickChange™ Site Directed Mutagenesis kit (StrataGene, San Diego, CA) was used to generate mutants with targeted amino acid substitutions. To introduce these specific amino acid substitutions, mutagenic primers (between 25 and 45 bases in length) were designed to contain the desired mutation that would result in the targeted amino acid substitution. Pfu DNA polymerase was then used to amplify both strands of the double-stranded vector, which contained the CBH I insertion sequence, with the resultant inclusion of the desired mutation from the synthetic oligonucleotides. Following temperature cycling, the product was treated with the exonuclease Dpn I to digest the parental methylated DNA template and the PCR product was used to transform Epicurian Coli XL1-Blue supercompetent cells.

The vector pPFE2/CBHI requires a relatively long PCR reaction (8.2 kB) to make site-specific changes using the Stratagene Quik Change protocol. The PCR reaction was optimized as follows using a GeneAmp PCR System 2400, Perkin Elmer Corporation. The reaction mixture contained 50 ng of template DNA, 125 ng each of the sense and antisense mutagenic primers, 5 µL of Stratagene 10X cloned *Pfu* buffer, 200 µM of each: dNTP, 5 mM $MgCl_2$ (total final concentration of $MgCl_2$ is 7 mM); and 2.5 U *Pfu* Turbo DNA polymerase. The PCR reaction was carried out for 30 cycles, each consisting of one minute denaturation at 96°C, 1 minute annealing at 69°C and a final extension for 10 min at 75°C, followed by a hold at 4°C. Agarose gel electrophoresis, ethidium bromide staining, and visualization under UV transillumination were used to confirm the presence of a PCR product.

PCR products were digested with the restriction enzyme Dpn1, to degrade un-mutagenized parental DNA, and transformed into *E. coli* (Stratagene Epicurian Coli Supercompetent XL-1 Cells). Ampicillin resistant colonies were picked from LB-amp100 plates and mutations were confirmed by DNA sequencing.

Template DNA from *E. coli* XL1-blue cells transformed with Dpn1 treated mutaginzed DNA was prepared for sequencing using the QIAprep-spin plasmid purification mini-prep procedure (Qiagen, Inc.). The transformed XL1-blue cells where grown overnight in 5 mL of LB broth with 100 µg/mL ampicillin selection. Cells were removed by centrifugation and the plasmid isolated using the protocol outlined in the QIAprep-spin handbook. The concentration of

the template DNA was adjusted to 0.25 µg/µL and shipped along with sequencing oligonucleotides to the DNA Sequencing Facility at Iowa State University.

After the mutation was confirmed by DNA sequence alignment comparisons using the software package OMIGA, and the DNA was prepared for transformation of *A. awamori*. The transformed *E. coli* XL1/blue cells were grown overnight on LB plates with 100 µg/mL ampicillin at 37°C. A single colony was then used to inoculate a 1 L baffled Erlenmeyer flask that contained 500 mL of LB broth and 100 µg/mL ampicillin. The culture was allowed to grow for 16 to 20 hours at 37°C with 250 rpm shaking in a NBS reciprocating shaking incubator. The cells were harvested and the plasmid DNA purified using a Promega maxi-prep purification kit. The purified maxi-prep DNA was subsequently used to transform *A. awamori* spheroplasts using the method described below.

Transformation of *Aspergillus awamori* with *Trichoderma reesei* CBHI coding sequence. Generating Fungal Spheroplasts.

A. awamori spheroplasts were generated from two-day-old cultures of mycelia pellets. A heavy spore suspension was inoculated into 50 mL of CM broth (5.0 g/L yeast extract; 5.0 g/L tryptone; 10 g/L glucose; 50 mL/L 20X Clutterbuck's salts, pH 7.5 (adjusted by addition of 2.0N NaOH)) and grown at 225 rpm and 28°C in a baffled 250 mL Erlenmeyer flask. The mycelia were collected by filtration through Miracloth and washed with ~200 mL KCM (0.7M KCl; 10mM MOPS pH 5.8). The washed mycelia were transferred to 50 mL of KCM + 500 mg Novazym 234 in a 50-mL unbaffled flask and incubated O/N at 80rpm and 30°C. After digestion, the remaining mycelia was removed by filtration through Miracloth and the spheroplasts were collected in 50 mL disposable tubes and pelleted at 2500 x g in a swinging bucket rotor for 15 minutes. The supernatant was discarded and the spheroplasts gently resuspended in 20 mL 0.7M KCl by tituration with a 25-mL disposable pipet. The spheroplasts were pelleted and washed again, then resuspended in 10 mL KC (0.7M KCl + 50mM $CaCl_2$). After being pelleted, the spheroplasts were resuspended into 1.0 mL of KC.

Transformation was carried out using 50 µL of spheroplasts + 5 µL DNA (pPFE1 or pPFE2 ~200 µg/mL) + 12.5 µL PCM (40% PEG8000 + 50mM CaCl2 + 10 mM MOPS pH 5.8). After incubation for 60 mins on ice, 0.5mL PCM was added and the mixture was incubated for 45 mins at room temperature. One milliliter of KCl was added and 370 µL of the mix was added to 10 mL

of molten CMK (CM + 2% agar + 0.7M KCl) top agar at 55°C. This mixture was immediately poured onto a 15mL CM170 plate (CM + 2% agar + 170µg/mL Zeocin). Negative transformation controls substituted sterile dH_2O for DNA. Plating the transformation mix onto CM plates without Zeocin performed positive spheroplast regeneration controls. The poured plates were incubated at 28°C in the dark for 2-7 days.

Transformation of *Aspergillus awamori* with native and modified CBH I coding sequence.

Aspergillus awamori spore stocks were stored at –70°C in 20% glycerol, 10% lactose. After thawing, 200 µL of spores were inoculated into 50 mL CM broth in each of eight-baffled 250 mL Erlenmeyer flask. The cultures were grown at 28°C, 225 rpm for 48 h. The mycelial balls were removed by filtration with sterile Miracloth (Calbiochem, San Diego, CA) and washed thoroughly with sterile KCM. Approximately 10 g of washed mycelia were transferred to 50 mL KCM + 250 mg Novozym234 in a 250 mL baffled Erlenmeyer flask. The digestion mixture was incubated at 30°C, 80 rpm for 1-2 h and filtered through Miracloth into 50 mL conical centrifuge tubes. The spheroplasts were pelleted at 2000xg for 15 min and resuspended in 0.7M KCl by gentle tituration with a 25 mL pipette. This was repeated once. After a third pelleting, the spheroplasts were resuspended in 10 mL KC, pelleted and resuspended in 0.5 mL KC using a wide-bore pipet tip. The washed spheroplasts were transformed by adding 12.5 µL PCM and 5 µL DNA (~0.5 µg/µL) to 50 µL of spheroplasts in sterile 1.5 mL Eppendorf tubes. After incubation on ice for 45 minutes, 0.5 mL of room temperature PCM was added to the transformation mixture and was mixed by tituration with a wide bore pipet tip. The mixture was incubated at room temperature for 45 minutes. One milliliter of KC was added and mixed. The mixture was allocated between four tubes of CM top agar at 55°C, which were each poured over a 15 mL CM170 plate. The plates were incubated at 28°C for 2-3 days. Subsurface colonies were partially picked with a sterile wide bore pipet tip, exposing the remaining part of the colony to air and promoting rapid sporulation. After sporulation, spores were streaked onto several successive CM100 or CM300 plates. After a monoculture was established, heavily sporulated plates were flooded with sterile spore suspension medium (20% glycerol, 10% lactose), the spores were suspended and aliquots were frozen at –70°C. Working spore stocks were stored on CM slants in screw cap tubes at 4°C. Protein production was confirmed and followed by western blot using anti-CBH I monoclonal antibodies and the Novex Western Breeze anti-mouse chromogenic detection kit (Novex, San Diego, CA). Extracting genomic DNA using the YeaStar Genomic DNA Kit (Zymo Research, Orange, CA) and carrying out PCR with *pfu*-turbo DNA polymerase (Stratagene, La Jolla) and *cbhI* primers confirmed insertion of the gene.

Production and purification of native rCBH I enzyme from *Aspergillus awamori.*

For enzyme production, spores were inoculated into 50 mL CM basal starch medium, pH 7.0, and grown at 32°C, 225 rpm in 250 mL baffled flasks. The cultures were transferred to 1.0 L of basal starch medium in 2800 mL Fernbach flasks and grown under similar conditions. For large-scale enzyme production (>1 mg), these cultures were transferred to 10 L basal starch medium in a New Brunswick BioFlo3000 fermenter (10-L working volume) maintained at 20% DO, pH 7.0, 25°C, and 300 rpm. The fermentation was harvested by filtration through Miracloth after 2-3 days of growth.

After further clarification by glass fiber filtration, the rCBH I protein was purified by passing the fermentation broth over four CBinD900 cartridge columns (Novagen, Madison, WI) connected in parallel using a Pharmacia FPLC System loading at 1.0 mL/min (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The cartridges were equilibrated in 20 mM Bis-Tris pH 6.5 prior to loading and washed with the same buffer after loading. The bound rCBH I was then eluted with 100% ethylene glycol (3 mL/column) using a syringe. Alternatively, the supernatant was passed over a para-aminophenyl β-D-cellobioside affinity column, washed with 100 mM acetate buffer, pH 5.0, 1mM gluconolactone and eluted in the same buffer containing 10mM cellobiose. In either method, the eluted rCBH I was concentrated in Millipore Ultrafree-15 spin concentrator with a 10kDa Biomax membrane to <2.0 mL and loaded onto a Pharmacia SuperDex200 16/60 size-exclusion column. The mobile phase was 20 mM sodium acetate, 100 mM sodium chloride, and 0.02% sodium azide, pH 5.0 running at 1.0 mL/min. The eluted protein was concentrated and stored at 4°C. Protein concentrations were determined for each mutant based upon absorbance at 280 nm and calculated from the extinction coefficient and molecular weight for each individual protein as determined by primary amino acid sequence using the ProtParam tool on the ExPASy website (http://www.expasy.ch/tools/protparam.html).

Clutterbuck's Salts (20X)

Na_2NO_3	120.0 g
KCl	10.4
$MgSO_4 \cdot 7H_2O$	10.4
KH_2PO_4	30.4

CM-

Yeast Extract-	5 g/L
Tryptone-	5 g/L
Glucose-	10 g/L
Clutterbuck's Salts-	50 mL

Add above to 900 mL dH_2O, pH to 7.5, bring to 1000 mL

CM Agar-CM + 20g/L Agar

CMK-CM Agar + 0.7M KCl

CM100-CM + 100 µg/mL Zeocin (Invitrogen, Carlsbad, CA)

CM170-CM + 170 µg/mL Zeocin, 15mL/plate

KCl-0.7M KCl

KC-0.7M KCl + 50 mM $CaCl_2$

KCM- 0.7M KCl + 10mM MOPS, pH 5.8

PCM-40% PEG 8000, 50 mM $CaCl_2$, 10 mM MOPS pH 5.8
(mix 4 mL 50% PEG + 0.5 mL 500 mM $CaCl_2$ stock + 0.5 mL 100 mM MOPS stock)

Basal Starch Medium-

Casein Hydrolysate, Enzymatic	5 g/L
NH_4CL	5 g/L
Yeast Extract	10 g/L
Tryptone	10 g/L
$MgSO_4 *7H_20$	2 g/L
Soluble Starch	50 g/L
Buffer (Bis-Tris-Propane)	50 mM
pH to 7.0 with NaOH	

Example 4. Production of Reduced Glycosylation rCBH I: Sites N270A; N45A; and N384A.

rCHI/pPFE2 has been optimized using site-directed mutagenisis to achieve expression of native molecular weight CBHI in *A. awamori* by the following ways. The QuickChange SDM kit (Stratagene, San Diego, CA) was used to make point mutations, switch amino acids, and delete or insert amino acids in the native *cbh1* gene sequence. The Quick Change SDM technique was performed using thermotolerant *Pfu* DNA polymerase, which replicates both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers. The procedure used the polymerase chain reaction (PCR) to modify the cloned *cbh1* DNA. The basic procedure used a supercoiled double stranded DNA (dsDNA) vector, with the *cbh1* gene insert, and two synthetic oligonucleotide primers containing a desired mutation. The oligonucleotide primers, each complimentary to opposite strands of the vector, extend during temperature cycling by means of the polymerase. On incorporation of the primers, a mutated plasmid containing the desired nucleotide substitutions was generated. Following temperature cycling, the PCR product was treated with a Dpn1 restriction enzyme. Dpn1 is specific for methylated and hemi-methylated

DNA and thus digests the unmutated parental DNA template, selecting for the mutation-containing, newly synthesized DNA. The nicked vector DNA, containing the desired mutations, was then transformed into *E. coli*. The small amount of template DNA required to perform this reaction, and the high fidelity of the *Pfu* DNA polymerase contribute to the high mutation efficiency and minimizes the potential for the introduction of random mutations. Three glycosylation-site amino acids on the pro surface were targeted for substitution of an alanine (A) residue in place of asparagines (N). Single site substitutions were successfully completed in the *cbh1* coding sequence at sites N45, N270, and N384, of Seq. ID NO: 4 by site-directed mutagenisis, and confirmed by DNA sequencing.

Double and triple combinations of this substitution have also been completed in the *cbh1* coding sequence at sites N45, N270, and N384 by site directed mutagenisis and confirmed by DNA sequencing. These double and triple site constructs also yield rCBHI enzymes with reduced glycosylation and, presumably, native activity.

Table 1.

Construct	Host	MW (kDa)	K_m μmol pNPL	V_{max} μmol pNP/min/mg protein
T. reesei	None	57.8	1.94	0.746
rCBHI wt cDNA	*A. awamori*	63.3	2.14	0.668
rCBHI wt genomic	*A. awamori*	63.3	--	--
rCBHI N270A	*A. awamori*	61.7	2.25	0.489
rCBHI N384A	*A. awamori*	61.3	--	--
rCBHI wt genomic (G)	*A. awamori*	63.3	--	--
rCBH I N45A	*A. awamori*	58.3	--	--
rCBHI N270/45A	*A. awamori*	58.3	--	--
rCBHI N384/270A	*A. awamori*	58.8		

As shown in Table 1, Western blot analysis of the supernatant, obtained from a single glycosylation site mutant CBHIN270A culture expressed in *A. awamori*, demonstrated that a decrease, to lower molecular weight (61.7 kDa), in the amount of glycosylation of the protein had occurred, as compared to that in the wild type cDNA (63.3 kDa), and the wild type genomic DNA (63.3 kDa). These results demonstrate a reduction in the level of glycosylation in the reduced glycosylation mutant CBHIN270A, via expression in A. awamori. It is also shown, in the Table, that the CBHIN270A enzyme nearly retained its native enzymatic activity when assayed using the *p*NPL substrate. The variants CBHIN45A and CBHI384A also demonstrate a reduction in amount of glycosylation and native activity when expressed from the heterologous host *A. awamori* and when combined in the double mutations CBHIN270/45A and CBHIN270/384A reduce the level of glycosylation further.

Example 5. Amino Acid Mutations Targeted To Improve Thermal Tolerance Of CBH I Helix Capping Mutants.

All α-helices display dipole moments, i.e. positive at N-terminal and negative at C-terminal. Compensation for such dipole moments (capping) has been observed in a number of protein structures [1, 2] and has been shown to improve the protein stability. For example, the

introduction of a negatively charged amino acid at the N-terminus and a positively charged amino acid at C-terminus of an α-helix increased the thermostability of T4 lysozyme[3] and hen lysozyme[4], via an electrostatic interaction with the "helix dipole." Five amino acid sites were identified for helix capping (see Table 5).

Peptide Strain Removal Mutants.

A small fraction of residues adopt torsion angles, phi-psi angles, which are unfavorable. It has been shown that mutation of such residues to Gly increased the protein stability as much as 4 kcal/mol. One amino acid site was selected for peptide strain removal (see Table 3).

Helix Propensity Mutants.

Two amino acid sites were selected for helix propensity improvement.

Disulfide Bridge Mutants.

Disulfide bonds introduced between amino acid positions 9 and 164 and between 21 and 142 in phage T4 lysozyme have been shown to significantly increase the stability of the respective enzymes toward thermal denaturation . The engineered disulfide bridge between residues 197 and 370 of CBH I should span the active site cleft and enhance its thermostability. The active site of CBH I is in a tunnel. The roof over the tunnel appears to be fairly mobile (high temperature-factors). At an elevated temperature the mobility of the tunnel is too significant to position all the active site residues. The disulfide linkage should stabilize the roof of the tunnel making the enzyme a consistent exocellulase even at a high temperature. Two amino acid sites were identified for new disulfide bridge generation.

Deletion Mutants.

Thermostable proteins have shorter loops that connect their structural elements than typical proteins. Our sequence alignment of CBH I, with its close homologs, suggests that the following residues may be deleted without significantly affecting its function. These loops exhibited high mobility as well. Three loops were identified, but these modifications were

considered high risk (buried hydrophobic regions may be exposed to solvent upon deletion of a natural loop) and will be saved for future work.

Proline Replacement Mutants.

The unique structure of proline dictates that fewer degrees of freedom are allowed around the alpha carbon that most other amino acids. The result of this structure is that peptides tend to loose flexibility in regions rich with proline. In order to assess possible sites for replacement of existing amino acids with proline, the phi/psi angles of candidate amino acid sites must conform with those consistent with proline. Each new site must also be evaluated for allowable side chain interactions and assurance that interactions with substrate are not altered. Seventeen amino acid sites were identified for proline replacement (See Table 2).

Example 6. Nucleic acid sequence of a variant exoglucance.

The present example demonstrates the utility of the present invention for providing a nucleic acid molecule having a nucleic acid sequence that has a sequence 5'-GGCGGAAAC*CCGCCT*GGCACCACC-3'. The identified nucleic acid sequence presents a novel linker region nucleic acid sequence that differs from previously reported nucleic acid sequence by the addition of one (1) codon. The invention in some aspects thus provides a nucleic acid molecule having a nucleic acid sequence that comprises a linker region of about 20 to 60 nucleotides identified here.

Table 2. Proline mutations to improve thermal tolerance.

Mutation	Native sequence and mutatgenic oligonucleotide
S8P – native sense strand	5'-GCACTCTCCAA**TCG**GAGACTCACCCG-3'
Mutagenic sense strand	5'-GCACTCTCCAA**CCG**GAGACTCACCCG-3'
Mutagenic anti-sense strand	5'-CGGGTGAGTCTC**CGG**TTGGAGAGTGC-3'
N27P – native sense strand	5'-GGCACGTGCACT**CAA**CAGACAGGCTCCG-3'
Mutagenic sense strand	5'-GGCACGTGCACT**CCA**CAGACAGGCTCCG-3'
Mutagenic anti-sense strand	5'-CGGAGCCTGTCTGT**GG**AGTGCACGTGCC-3'
A43P – native sense strand	5'-GGCGCTGGACTCAC**GCT**ACGAACAGCAGCACG-3'
Mutagenic sense strand	5'-GGCGCTGGACTCAC**CCT**ACGAACAGCAGCACG-3'
Mutagenic anti-sense strand	5'-CGTGCTGCTGTTCGT**AGG**GTGAGTCCAGCGCC-3'
G75P – native sense strand	5'-GCTGTCTGGAC**GGT**GCCGCCTACGCG-3'
Mutagenic sense strand	5'-GCTGTCTGGAC**CCT**GCCGCCTACGCG-3'
Mutagenic anti-sense strand	5'-CGCGTAGGCGGC**AGG**GTCCAGACAGC-3'
G94P – native sense strand	5'-GCCTCTCCATT**GGC**TTTGTCACCC-3'
Mutagenic sense strand	5'-GCCTCTCCATT**CCC**TTTGTCACCC-3'
Mutagenic anti-sense strand	5'-GGGTGACAAA**GGG**AATGGAGAGGC-3'
E190P – native sense strand	5'-GGCCAACGTT**GAG**GGCTGGGAGCC-3'
Mutagenic sense strand	5'-GGCCAACGTT**CCG**GGCTGGGAGCC-3'
Mutagenic anti-sense strand	5'-GGCTCCCAGCC**CGG**AACGTTGGCC-3'
S195P – native sense strand	5'-GGCTGGGAGCCG**TCA**TCCAACAACGCG-3'
Mutagenic sense strand	5'-GGCTGGGAGCCG**CCA**TCCAACAACGCG-3'
Mutagenic anti-sense strand	5'-CGCGTTGTTGGA**TGG**CGGCTCCCAGCC-3'
K287P - native sense strand	5'-CGATACCACCAAG**AAA**TTGACCGTTGTCACCC-3'
Mutagenic sense strand	5'-CGATACCACCAAG**CCA**TTGACCGTTGTCACCC-3'
Mutagenic anti-sense strand	5'-GGGTGACAACGGTCAA**TGG**CTTGGTGGTATCG-3'
A299P - native sense strand	5'-CGAGACGTCGGGT**GCC**ATCAACCGATAC-3'
Mutagenic sense strand	5'-CGAGACGTCGGGT**CCC**ATCAACCGATAC-3'
Mutagenic anti-sense strand	5'-GTATCGGTTGAT**GGG**ACCCGACGTCTCG-3'
Q312P/N315P - native sense strand	5'-GGCGTCACTTTC**CAG**CAGCCC**AAC**GCCGAGCTTGG-3'
Mutagenic sense strand	5'-GGCGTCACTTTC**CCG**CAGCCC**CCC**GCCGAGCTTGG-3'
Mutagenic anti-sense strand	5'-CCAAGCTCGGC**GGG**GGGCTG**CGG**GAAAGTGACGCC-3'
G359P - native sense strand	5'-GGCTACCTCT**GGC**GGCATGGTTCTGG-3'
Mutagenic sense strand	5'-GGCTACCTCT**CCC**GGCATGGTTCTGG-3'
Mutagenic anti-sense strand	5'-CCAGAACCATGCC**GGG**AGAGGTAGCC-3'

S398P/S401P- native sense strand	5'-GCGGAAGCTGC**TCC**ACCAGC**TCC**GGTGTCCCTGC-3'
mutagenic sense strand	5'-GCGGAAGCTGC**CCC**ACCAGC**CCC**GGTGTCCCTGC-3'
mutagenic anti-sense strand	5'-GCAGGGACACC**GGG**GCTGGT**GGG**GCAGCTTCCGC-3'
A414P - native sense strand	5'-GTCTCCCAAC**GCC**AAGGTCACC-3'
mutagenic sense strand	5'-GTCTCCCAAC**CCC**AAGGTCACC-3'
mutagenic anti-sense strand	5'-GGTGACCTT**GGG**GTTGGGAGAC-3'
N431P/S433P - native sense strand	5'-GGCAGCACCGGC**AAC**CCT**AGC**GGCGGCAACCC-3'
mutagenic sense strand	5'-GGCAGCACCGGC**CCC**CCT**CCC**GGCGGCAACCC-3'
mutagenic anti-sense strand	5'-GGGTTGCCGCC**GGG**AGG**GGG**GCCGGTGCTGCC-3'

Table 3. Mutation to remove peptide strain.

Mutation site	Native sequence and mutatgenic oligonucleotide
S99G- native sense strand	5'-GGCTTTGTCACCCAG**TCT**GCGCAGAAGAACGTTGGC-3'
mutagenic sense strand	5'-GGCTTTGTCACCCAG**GGT**GCGCAGAAGAACGTTGGC-3'
mutagenic anti-sense strand	5'-GCCAACGTTCTTCTGCGC**ACC**CTGGGTGACAAAGCC-3'

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Table 3b. Y245G analogs to remove product inhibition.

Mutation site	Native sequence and mutatgenic oligonucleotide
R251A – native sense strand	5'-CCGATAAC**AGA**TATGGCGGC-3'
mutagenic sense strand	5'-CCGATAAC**GCC**TATGGCGGC-3'
mutagenic anti-sense strand	5'-GCCGCCATA**GGC**GTTATCGG-3'
R394A– native sense strand	5'-CCCGGTGCCGTG**CGC**GGAAGCTGCTCCACC-3'
mutagenic sense strand	5'-CCCGGTGCCGTG**GCC**GGAAGCTGCTCCACC-3'
mutagenic anti-sense strand	5'-GGTGGAGCAGCTTCC**GGC**CACGGCACCGGG-3'
F338A– native sense strand	5'-GCTGAGGAGGCAGAA**TTC**GGCGGATCCTCTTTCTC-3'
mutagenic sense strand	5'-GCTGAGGAGGCAGAA**GCC**GGCGGATCCTCTTTCTC-3'
mutagenic anti-sense strand	5'-GAGAAAGAGGATCCGCC**GGC**TTCTGCCTCCTCAGC-3'
R267A– native sense strand	5'-GGAACCCATAC**CGC**CTGGGCAACACCAGC-3'
mutagenic sense strand	5'-GGAACCCATAC**GCC**CTGGGCAACACCAGC-3'
mutagenic anti-sense strand	5'-GCTGGTGTTGCCCAG**GGC**GTATGGGTTCC-3'
E385A– native sense strand	5'-CCTACCCGACAAAC**GAG**ACCTCCTCCACACCCGG-3'
mutagenic sense strand	5'-CCTACCCGACAAAC**GCC**ACCTCCTCCACACCCGG-3'
mutagenic anti-sense strand	5'-CCGGGTGTGGAGGAGGT**GGC**GTTTGTCGGGTAGG-3'

Table 4. N to A mutations to remove glycosylation.

Mutant	**Native sequence and mutagenic oligonucleotide**
N45A - native sense strand	5'-GGACTCACGCTACG**AAC**AGCAGCACGAACTGC-3'
mutagenic sense strand	5'-GGACTCACGCTACG**GC**CAGCAGCACGAACTGC-3'
mutagenic anti-sense strand	5'-GCAGTTCGTGCTGCT**GGC**CGTAGCGTGAGTCC-3'
N270A - native sense strand	5'-CCCATACCGCCTGGGC**AAC**ACCAGCTTCTACGGCCC-3'
mutagenic sense strand	5'-CCCATACCGCCTGGGC**GC**CACCAGCTTCTACGGCCC-3'
mutagenic anti-sense strand	5'-GGGCCGTAGAAGCTGGT**GGC**GCCCAGGCGGTATGGG-3'
N384A - native sense strand	5'-GGACTCCACCTACCCGACA**AAC**GAGACCTCCTCCACACCCG-3'
mutagenic sense strand	5'-GGACTCCACCTACCCGACA**GCC**GAGACCTCCTCCACACCCG-3'
mutagenic anti-sense strand	5'-CGGGTGTGGAGGAGGTCTC**GGC**TGTCGGGTAGGTGGAGTCC-3'

Table 5. Helix capping mutations to improve thermal tolerance.

Mutant	Native sequence and mutagenic oligonucleotide
E337R - native sense strand	5'-GCTGAGGAGGCA**GAA**TTCGGCGG-3'
mutagenic sense strand	5'-GCTGAGGAGGCA**CGC**TTCGGCGG-3'
mutagenic anti-sense strand	5'-CCGCCGAA**GCG**TGCCTCCTCAGC-3'
N327D - *native sense strand*	5'-GGCAACGAGCTC**AAC**GATGATTACTGC-3'
mutagenic sense strand	5'-GGCAACGAGCTC**GAC**GATGATTACTGC-3'
mutagenic anti-sense strand	5'-GCAGTAATCATC**GTC**GAGCTCGTTGCC-3'
A405D - *native sense strand*	5'-CCGGTGTCCCT**GCT**CAGGTCGAATCTCAGTCTCCC-3'
mutagenic sense strand	5'-CCGGTGTCCCT**GAT**CAGGTCGAATCTCAGTCTCCC-3'
mutagenic anti-sense strand	5'-GGGAGACTGAGATTCGACCTG**ATC**AGGGACACCGG-3'
Q410R - *native sense strand*	5'-GCTCAGGTCGAATCT**CAG**TCTCCCAACGCC-3'
mutagenic sense strand	5'-GCTCAGGTCGAATCT**CGC**TCTCCCAACGCC-3'
mutagenic anti-sense strand	5'-GGCGTTGGGAGA**GCG**AGATTCGACCTGAGC-3'
N64D - *native sense strand*	5'-CCCTATGTCCTGAC**AAC**GAGACCTGCGCG-3'
mutagenic sense strand	5'-CCCTATGTCCTGAC**GAC**GAGACCTGCGCG-3'
mutagenic anti-sense strand	5'-CGCGCAGGTCTC**GTC**GTCAGGACATAGGG-3'
N64D - *native sense strand*	5'-GCTCGACCCTATGTCCTGAC**AAC**GAGACCTGCGCGAAGAACTGC-3'
mutagenic sense strand	5'-GCTCGACCCTATGTCCTGAC**GAC**GAGACCTGCGCGAAGAACTGC-3'
mutagenic anti-sense strand	5'-GCAGTTCTTCGCGCAGGTCTC**GTC**GTCAGGACATAGGGTCGAGC-3'

Legend for Tables 2, 3, 3b, 4 and 5. Amino acid mutations sites are listed in the left column. The first letter in the designation is the amino acid of the native protein based upon IUPAC convention for one-letter codes for amino acids. The number represents the amino acid location as designated from the start of the mature protein (excluding the signal peptide, i.e. QSA...). The letter designation after the number represents the amino acid that will occur as a result of the mutation. For example N64D represents the asparagine at site 64 changed to an aspartic acid. The native sense strand sequence for each site is listed in the right column with the oligonucleotide primers (sense and anti-sense) used to obtain the desired mutation below the native sequence in each case. In addition the codon for the targeted amino acid is bolded and the nucleotide substitutions in the mutagenic primers underlined. In some cases only one nucleotide

substitution was required the make the desired change, and in others 2 or 3 substitutions were required. In a few cases, double mutations were made with a single mutagenic oligonucleotide.

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